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Cytotoxic Effect of *Peganum harmala* L. Extract and Induction of Apoptosis on Cancerous Cell Line

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Abstract:

The methanol extract of Peganum harmala L. was tested in vitro on the cancercell line Hep-2 (human laryngeal Carcinoma Cell, the results revealed dose- dependant significant differences, there was increasing cytotoxic effect at concentrations 156-10000 µg/ml. At the first 24 hrs. of exposure time, and with no significant differences on all period time (24, 48 and 72 hrs.). And the results showed that was increasing on apoptotic process after treated with methanol extract of P. harmala to repairing the damage of the cell and induction of cell death compared with control (not treated) on concentration 156 and 312 µg/ml.

Introduction:

P. harmala is a wild-growing flowering plant belonging to the Zygophylaceae family and is found abundantly in Middle East and North Africa (1). This plant is known as “Espand” in Iran, “Harmal” in North Africa and “African Rue”, “Mexican Rue” or “Turkish Rue” in united state. The pharmacologically active compounds of P. harmala are several alkaloids, which are found especially in the seed and the roots. These include β- carbolines such as: Harmline, harmaline (identical with harmidine), harmalol and Harman and quinazoline derivatives: vasicine. The alkaloidal content of the unripe seed is less than the ripe ones (2).

From ancient time, peganum harmala was claimed to be an important medicinal plant. Its seeds were known to possess hypothermic and essentially hallucinogenic properties. Various authors have undertaken studies on the antibacterial, antifungal and antiviral effects of Peganum harmala seeds, and used as antitumor (3).

Cells exposed to DNA- damaging agents such as ionizing radiation, UV radiation, and chemical agents initiate a complex response that includes the inhibition of cell cycle progression until damage is repaired. If the DNA damage is beyond repair, cells may enter a prolonged state of arrest or undergo a programmed cell death known as apoptosis, thereby maintaining genetic stability in the organism (4).

Apoptosis is a naturally occurring cellular event that helps maintain organism homeostasis. A cell can trigger
apoptosis in a neighboring cell through signaling processes such as Fas surface receptor (CD95 or APO1) and Fas-Ligand (cytokines, interferons, interleukins) interaction (5).

Apoptosis is a tightly regulated and at the same time highly efficient cell death program which requires the interplay of a multitude of factors. The components of the apoptotic signalling network are genetically encoded and are considered to be usually in place in a nucleated cell ready to be activated by a death-inducing stimulus. Apoptosis can be triggered by various stimuli from outside or inside the cell, e.g. by ligation of cell surface receptors, by DNA damage as a cause of defects in DNA repair mechanisms, treatment with cytotoxic drugs or irradiation, by a lack of survival signals, contradictory cell cycle signalling or by developmental death signals. Death signals of such diverse origin nevertheless appear to eventually activate a common cell death machinery leading to the characteristic features of apoptotic cell death. (6 and 7).

Material and Methods:

This study was carried out on the Iraqi Center of Cancer and Medical Genetic Researches (ICCMGR) at 2009- 2010.

Preparation of methanol extracts:

The seeds of Peganum harmala were collected from local markets of Baghdad- Iraq during 2008-2009. According to Saleem et al. (8) 10 gm of dried seeds were soaking in 100 ml of 80% methanol for 24 hrs., then crashed by mortar at room temperature. The mixture were continuously starrier for 1 hrs., and filtered at Watmann filter paper -1-, The extract were evaporator to dryness at 30 ˚C.

1 g. of the powder extract dissolved in 100 ml Serum free media (SFM) as solvent, the suspension then sterilize by filtered (0.22 M.).

Reagents for Chemical Tests:

Reagents for chemical testes were used to detect active (secondary metabolites) compound of methanol extract to plant parts. The extract tested by the fallowing reagents:-

- Test of Alkaloids (mayers and Dregendroffs reagent).
- Test of Terpenoids- Saponins (Saponins, HgCl2 reagent) respectively.
- Test of phenoles (Tannins and Flavonoids) (Fecl3 and KoH reagents). (9, 10 and 11).

Cell Line and Culture:

This in vitro method was used to investigate the effect of the methanol extract of P. harmala on Human laryngeal carcinoma (Hep-2) cell line. The origin and description of this cell line was mentioned by Moore et al. (12). It is a human laryngeal carcinoma excised from 57 years old man, then transplanted in immune suppressed rat by cortisone. After growth of the tumor in the rat, it was then excised and transplanted as an in vitro tissue culture. Passages 237 of Hep-2 cell line was used throughout this study and cells were maintained using RPMI- 1640 medium (Rosswell Park Memorial Institute) (Sigma chemicals) and supplemented with 10% of Fetal Bovine Serum (FBS).

Cell line Preparation and Cytoxicity assay:

The growth medium was decanted off. Tow to three ml of trypsin- versene was added to the cell sheet and the flask recked gently. After approximately 30 seconds most of the tyrosine- versene was poured off and the cells incubated at 37 ˚C until they had detached from the flask. Cells were further dispensed by pipetting in growth medium (13). Afterwards, 200 µl of cells in growth medium were added to each well of sterile 96- well microtitration plate. The plates were sealed with a self adhesive film, lid placed on and incubated at 37 ˚C.

When the cells are in exponential growth, i.e. after Lag phase, the medium was removed and serial dilutions of methanol extract in serum free media (SFM) (10000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.0625 or 0.0 µg/ml) were added to the wells. 3 replicates were used for each concentration of each extracts. Afterwards, the plates were re-incubated at 37 ˚C. For the selected exposure times (24, 48 or 72 hrs.).

Supernatants were removed from the wells of the microtitration plate at the end of each exposure period while maintaining sterile conditions. 100 µ l of MTT (3- (4, 5- Dimethlthiazol- 2- yl) – 2,5-diphenyltetrazolium bromide, atetrazole) solution (1mg\ml) and 50 µ l of SFM in (2: 1 v/ v) was added to each of the wells in the microtitration plate, and then covered incubated for 4 hrs. at 37 ˚C. At the end of this incubation period 200 µ l of Dimethyl Sulfocside (DMSO) was added to each well to dissolve the formazan crystals. The Optical density was determined at 550 nm. using ELISA reader (13).

Detecting apoptosis:

After finding medium growth inhibitory concentrations (IC50) by Cytotoxicity assay (that was µg/ml after 24 hrs. from the period of exposure time). The cell line was cultured on tissue culture slide chumber, then after 24 hrs. the cell exposured to 312, 156, 78 and 0.0 µg/ml of methanol extract of P. harmala for 24 hrs.. Then for study the apoptotic pathway we used terminal dUTP nick-end labeling [Tunel Apoptosis Detection Kit (DNA Fragmentation\ Fluorescence Staining) BioAssay, USBiological, Cat: T9162-80].

During apoptosis, the chromatin condenses against the nuclear envelope, and the DNA inside the nucleus becomes fragmented. Terminal Deoxynucleotidyl Transferase, when used with a fluorescent marker, allows detection of cells with apoptotic DNA fragmentation. TdT is an enzyme that catalyzes the repetitive addition of dNTPs to the 3´-OH end of a DNA fragment. In TUNEL analysis, fluorescently

conjugated dUTPs are added to the 3´-OH groups of the DNA fragments, making the apoptotic cells visible by fluorescent microscopy.

Statistics:

Experiments data were analyzed using statistical Last Significant Design (LSD). Significance between control and samples was determined using students f- test. A P value ≤ 0.05 was considered statistically significant.

Results:

Chemical Tests

The result shows the chemical tests for the general constituents of the aqueous and methanol extracts of P. harmala. The methanol extract contained Alkaloid, Saponines & Flavenoids. Wherase the aqueous extract contained Terpenoids, Saponines, Tannins, Flavonoids (Table 1).

Table 1. Phytochemicals detected in the crude extracts of P. harmala

Phytochemicals to be detected		Methanol extract
Alkaloids	Mayers regent	+
	Dragendroffs regent	+
Terpenoids	Saponins	+
	$Hgcl_2$	-
phnoles	Tannins/ $Fecl_3$	-
	Flavonoids/ KoH	+

+ : The extract contains the designated phytochemical

- : The extract not contains the designated phytochemical

Cytoxicity assay:

• The effect of methanol extract of P. harmala on Hep-2 cell line at different concentrations (10000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.0625 or 0.0 µg/ml) and with different periods time (24, 48 and 72 hrs.) were tested (Fig 1 and 2). The result shows that was a significant effect at level (P < 0.05) between concentrations on methanol extract started on 78- 10000 µg/ml compared with control (0.0 µg/ml) with no significant difference at level (P > 0.05) on 156-10000 µg/ml at the first 24 hrs. and with all the concentration at 48 and 72 hrs. compared with control (0.0 µg/ml). The results revealed dose- dependant significant differences, there was increasing cytotoxic effect at concentration with highest inhibitory concentration was 93% at concentrations 1250 and 625 µg/ml at 72 hrs. on methanol extract.

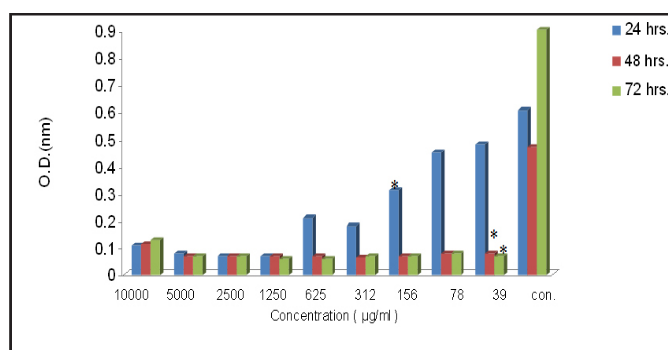
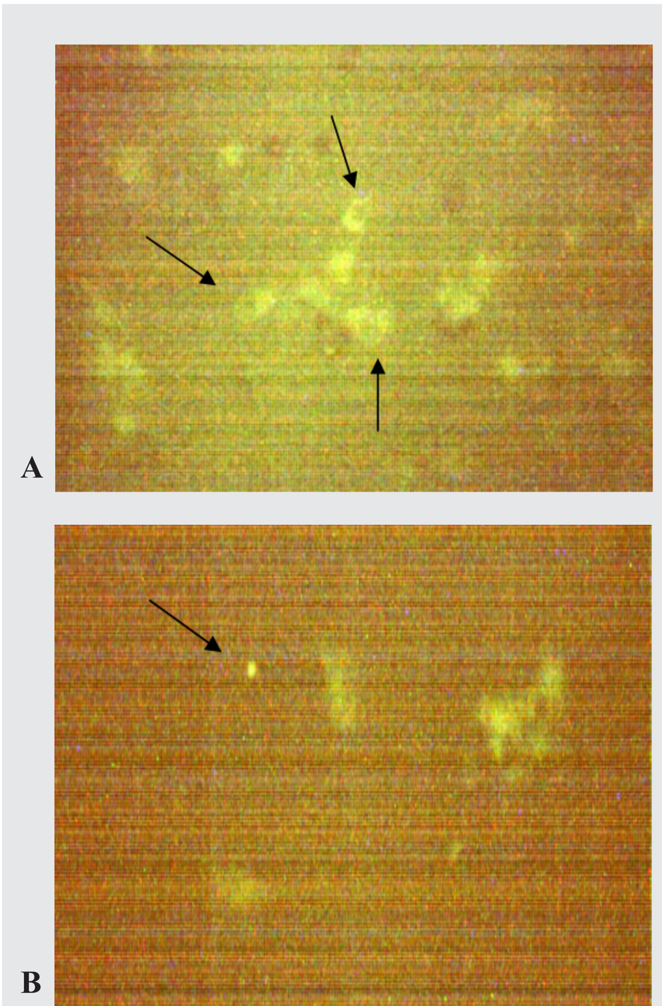


Figure (2): effect of methanol extracts of P. harmala on (Hep-2) cell line after 24, 48 and72 hrs.
** refer to start significant effect at p>0.05.*

Detecting apoptosis:

• Cell death in adherent Hep-2 cell line after treatment with methanolic extract of P. harmala was result of apoptosis as determined by Tunel assay (Picture 1). The result shows that was a significant effect at level (P<0.05) between concentrations on the early stage of apoptotic percentage with highest percentage (60%) on concentrations 156 and 312 µg/ml who were show no significant difference at level (P > 0.05) between them compared with control (0.0 µg/ml). While there was no significant difference at level (P > 0.05) between all concentrations compared with control (0.0 µg/ml) on the late stage of apoptotic percentage (Fig. 3). There was increasing of apoptotic effect with highest inhibitory concentration on methanol extract of P. harmala.



Picture (1): effect of methanol extracts of P. harmala on cell death by immunofluorescence using Tunel assay shows: (A) the early (B) the late stage.

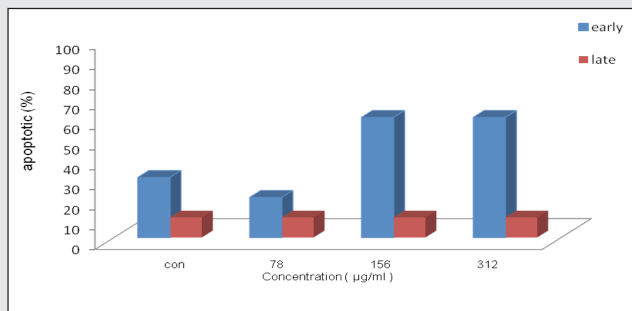


Figure (3): effect of methanol extracts of P. harmala on cell death by immunofluorescence using Tunel assay.

Discussion:

The results shows that the aqueous extract of P. harmala absence from alkaloids except pyrolides alkaloids, whereas the methanol extract contain alkaloids, that refer to the methanol extraction batter than aqueous in alkaloids that was the major content of the plant, the active alkaloids of harmala seeds are the MAOI- A (Mono Amine Oxidase Inhibitor) compounds: harmane, harmine, harmaline, harmalol, tetrahydroharmine, vasicine and vasicine. And the total harmala alkaloids are at least 5.9% per dried weight, wherase the stem of the plant contain about 0.36% alkaloids, the leaves about 0.52%, and roots upto 2.5% (14). Results obtained indicate that alkaloids of Peganum have a high cell toxicity in vitro, where varing concentrations (10-120 micrograms\ ml) of total alkaloid extracts of Peganum harmala seeds on four tumoural cell lines: Med- med, Ucp- med carcinoma, Ucp- med sarcoma and Sp2\ O- Agl4 for 24 hrs. (15).

Mechanism of the cytotoxic activity of P. harmala seeds extract was the main motivation of this investigation. Previous work (16) has shown that one of the compounds of the plant (B- carbolines) could intercalate in to DNA in theory, this effect may cause inhibition of DNA topoisomerases and results in cytotoxicity. The most accepted explanation for the cytotoxic effect of plant extract is the ability of plants to induce the programmed cell death in cancerous cells, as attempt to arrest their proliferation. Amumber of food items as well as herbal medicine have been reported to produce toxic effects by inducing programmed cell death (17).

In tissue culture cell lines, the cytotoxic drugs induce the molecular regulater of physiological apoptosis (18). Several mechanisms have been identified to underlie the modulation of programmed cell death by plants including endonuclease activation, involvement of p53, activation of caspase- 3 protease via a Bcl- 2 insensitive pathway, potentiate free radical formation and accumulation of sphinine (19).

Tough the mode of action is still unclear, the observed Cytotoxicity can be attributed to the harmala alkaloids present in the tested fraction, In

fact, it is known that harmalol (an indole alkaloid from P. harmala) in habited the proliferation of K562-Leukaemic tumor cell line at concentrations of 10 µg/ml by inhibiting DNA synthesis and cell division (20).

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التأثير السمي وتحفيز الموت المبرمج لمستخلص نبات الحرمل *Peganum harmala .L* على الخطوط السرطانية

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الخلاصة:

تم اختبار تأثير السمية الخلوية للمستخلص الميثانولي لنبات الحرمل *P. harmala* خارج الجسم الحي على الخط السرطاني Hep-2. اظهرت النتائج وجود تأثير معنوي للتراكيز المستخدمة، اذ لوحظ زيادة السمية الخلوية باستخدام التراكيز (156- 10000) مايكروغرام/ مل وخلال الـ 24 ساعة الاولى من تعريض الخلايا مع عدم وجود فروقات معنوية خلال فترات التعريض الثلاثه (48 ،24 و72 ساعة). كما اظهرت النتائج النتائج وجود زيادة في عملية الموت المبرمج بعد المعاملة بالمستخلص لاصلاح الضرر الخلوي عن طريق التحفيز على الموت الخلوي مقارنة بمعاملة السيطرة)غير المعاملة بالمستخلص(وللتراكيز 156 و 312مايكروغرام/ مل المستخدمة قيد الدراسة.